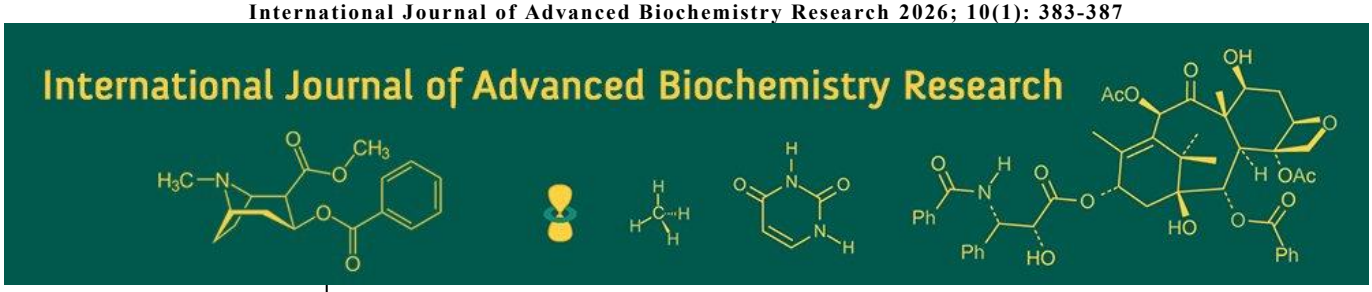

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Effect of fucoidan extracted from the brown seaweed, *Cystoseira indica* on growth, survival, immune response and disease resistance to *Vibrio parahaemolyticus* in *Penaeus vannamei* (BOONE, 1931)

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Abstract
Background: A 60-day experiment was conducted to evaluate the fucoidan effect of *Cystoseira indica* on the growth performance, survival, immune response and disease resistance to *Vibrio parahaemolyticus* in *Penaeus vannamei* at College of Fisheries Science, Kamdhenu University, Veraval - 362 265, Gujarat, India.
Methods: The study used three different *Cystoseira indica* fucoidan (CIF) extracted diets *viz*. T_1(0.5% CIF), T_2 (1% CIF) and T_3 (2% CIF) along with the control treatment to the larvae of *Penaeus vannamei* with a stocking density 20 numbers/tank of average body weight 0.012g were stocked in 60 L capacity rectangular plastic tanks. Each treatment were kept in triplicate factor followed the completely randomized design (CRD).
Results: *P. vannamei* expressed a better growth performance in terms of WG, SGR, FCR, PER and immune response in terms of PO and NBT. A lower mortality was observed in T_3 (2% CIF) diet. Based on the results, it can be concluded that T_3 (2% CIF) diet showed the better growth, immune response and disease resistance to *Vibrio parahaemolyticus* suspension (1×10^7 cfu ml^{-1}) in *Penaeus vannamei*.

Keywords: *Cystoseira indica*, Growth performance, *Penaeus vannamei*, *Vibrio parahaemolyticus*

Introduction
Aquaculture is cultivating or rearing aquatic organisms like finfishes, molluscs, crustaceans and aquatic plants (Huntingford *et al.,* 2012) [14]. In aquaculture, cultured shrimps are among the most economically and nutritionally important products. Due to their high acceptance in global markets, they also help developing countries earn revenue in dollar terms (Bondad-Reantaso *et al.,* 2012) [4]. In 2020, white-leg shrimp *(Penaeus vannamei*) contributed 51.7% to total crustacean aquaculture with a total production of 5.8122 million tonnes (FAO, 2022). White-leg shrimp dominated the crustacean aquaculture sector because of their benefits, which include their high stocking density, high growth rate, resistance to a wide range of salinities and temperatures, and lower dietary protein needs than *P. monodon* (Briggs *et al.,* 2004) [5].
Diseases have posed serious obstacles to the shrimp farming sector, making it more difficult to grow and sustain. A wide variety of pathogens, including bacteria, viruses, fungi, and parasites, can infect shrimp. The primary problem was viral diseases, namely White Spot Syndrome Virus (WSSV), which caused farmers to suffer large losses (Lightner *et al.,* 2012) [17]. Disease outbreaks that may lead to mass mortality are the most discussed constraints in shrimp aquaculture. In 2016, the economic loss due to disease outbreaks was as high as USD 6 billion globally (Rizan *et al.,* 2018) [22]. It was found that strains of Vibrio, for example, *Vibrio parahaemolyticus*, are the causative agents for the Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) that have resulted in severe economic losses in shrimp farming worldwide (Tran *et al.,* 2013) [34].

The use of antibiotics, vaccines, and immunostimulants are the most common methods to prevent disease outbreaks (Apines-Amar and Amar, 2015) [2]. The downside of using antibiotics in aquaculture is that they disrupt aquatic microflora, cause harmful residues to build up in aquatic organisms, and contribute to the emergence of drug-resistant bacteria and pathogens (Singer *et al.,* 2019) [24]. Vaccination also offers a potential solution for disease prevention. Scientists have been exploring ways to strengthen shrimp immunity with a Vibrio "vaccine" since the early 1990s. The discovery of an alternative adaptive immune system in invertebrates, including shrimp, has made this approach even more promising. However, developing a vaccine that's ready for commercial use isn't a quick or easy process. Shrimp have a unique immune system that researchers are still working to fully understand, making vaccine development challenging (Amatul-Samahah *et al.,* 2020) [1].

The use of natural compounds in aquaculture possesses a variety of advantages i.e. easier biodegradability, environmentally friendly, and less likely to contribute to antimicrobial resistance. The cost of the use of immunostimulants is also affordable, thus they are a potential treatment for the disease in shrimp farms (Smith *et al.,* 2003; dos Santos Filho *et al.,* 2023) [27, 9].

Macroalgae, commonly known as seaweeds, are multicellular, photosynthetic organisms that are found in almost all aquatic environments worldwide (Dillehay *et al.,* 2008) [8]. Due to the presence of bioactive compounds such as laminarin, fucoidan, carrageenan, and alginate,they are considered to be a potential immunostimulant for the resistance against disease in aquaculture. Fucoidan, a sulfate and L-fucose-rich polysaccharide has gained a lot of attention globally, especially in the food and pharmaceutical industries, due to its potential therapeutic applications. Its significant biological effects are attributed to its distinctive structure. Fucoidan has been known to possess antioxidant, anticancer, anticoagulant, antithrombotic, immunomodulatory, antiviral, and anti-inflammatory properties (Luthuli *et al.,* 2019) [19]. Fucoidan was found to increase the immune response in white-leg shrimp by activation of prophenoloxidase (proPO) and haemocyte degranulation which resulted in enhanced resistance against *V. alginolyticus* infection. In a study by Sudaryono *et al.,* (2018) [29] oral administration of hot water extract from Brown Seaweed, *Sargassum cristaefolium* was found to increase immune response in white-leg shrimp and resistance against *vibrio parahaemolyticus*. Fucoidan extract from brown seaweed, *Cystoseira trinodis* as a feed-additive in *Litopenaeus vannamei* enhanced growth, immune response and disease resistance against WSSV (Salehpour *et al.,* 2021) [23]. Moreover, the fucoidan from *Sargassum wightii* enriched with artemia nauplii reduced WSSV-induced mortality in *Penaeus monodon* postlarvae (Madasamy *et al.,* 2012) [20]. Thus, numerous studies support the positive effect of fucoidan extract on innate immunity and disease resistance against pathogens in shrimps.

Materials and Methods

The brown seaweed *Cystoseira indica* was collected from the rocky shore areas along the Veraval coast in Gujarat, India. The collected seaweed was washed thoroughly and allowed them to dry in sun-shade. After drying, a mixer grinder was used to grind it and fibrous material was removed by passing the dried mixture through a mesh sieve.

A fucoidan extraction was obtained following the method described by Yang *et al*. (2008). A fucoidan extraction from the brown seaweed was obtained the method described by (Patel *et al.,* 2025) [21]. The fucoidan yield was calculated based on the following formula:

$$Yield(\%) = \frac{Weight\ of\ obtained\ fucoidan\ (g)}{Weight\ of\ dried\ biomass\ (g)} \times 100$$

An experimental animal, white-leg shrimp (*Penaeus vannamei*) post larvae with an average weight of 0.012 g were were brought from a commercial shrimp hatchery, Gujarat. An oxygenated post larvae in polythene bags were stocked in plastic tanks of 500 L capacity and fed a commercial diet for 7 days to acclimatize them to the experimental conditions.

Four experimental groups in triplicate manner were listed as follows: Control (T_0) (Basal diet with no fucoidan extract), T_1 (0.5% *C. indica* fucoidan extract), T_2 (1% *C. indica* fucoidan extract), and T_3 (2% *C. indica* fucoidan extract) with 35% protein level were formulated (Hardy 1980). A uniform sized post-larvae were stocked at 20 numbers/ tank. The experiment was carried out for 60 days were fed two times a day at the rate of 10% of body weight.

The ingredients used in the formulation of different experimental diets were fishmeal, rice bran, groundnut oil cake, tapioca flour, vitamin, and mineral premix. All these were purchased from the local market. The composition of the experimental diet was given in Table 1. The required quantities of ingredients were weighed accurately, mixed, and hand-kneaded to require consistency with just a sufficient quantity of water to get smooth dough. The prepared dough was cooked under steam in an autoclave at 121°C and 15 lbs. pressure for 10-15 minutes. The cooked feed was cooled to room temperature rapidly by spreading in an enamel tray and the required dose of fucoidan extract and vitamin-mineral premix were added, mixed, and blended. The dough was extruded through a pelletizer having a 2 mm diameter. The pellets were spread on a plastic sheet and sun-dried till the moisture content was reduced to less than 10%. Diets were packed separately in high-density polythene bags, labeled, and stored on a wooden shelf at room temperature for further use.

Table 1: Composition of experimental diets

	Diets (35% Protein)			
Ingredient (%)	T_0	T_1	T_2	T_3
Fishmeal	48	48	48	48
Wheat flour	14	13.5	13	12
GNOC	18	18	18	18
Tapioca powder	10	10	10	10
C. indica fucoidan extract	0	0.5	1	2
Fish oil	4	4	4	4
Plant oil	4	4	4	4
Vitamin & minerals	2	2	2	2
Total	100	100	100	100

The growth performance of shrimp was assessed in terms of weight attained, specific growth rate (SGR%/day), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate (%) were calculated using the standard formulae El-Sayed (1999) [10].

At the end of the feeding trial, the animals were used to measure immune parameters and resistance to *Vibrio*

parahaemolyticus. A 100 µl haemolymph was collected from the ventral sinus of 10 shrimps of all treatments into a 1 ml syringe (26 gauge) containing 900 µl anticoagulants solution (0.114 M trisodium citrate and 0.1 M sodium chloride, pH 7.45) for analysis of prophenoloxidase activity, respiratory burst activity and total haemocyte count. A phenoloxidase (PO) assay was performed as described by López *et al.* (2003) [18]. A respiratory burst activity (NBT) assay was performed as described by Song and Hsieh (1994) [28]. A total haemocyte count (THC) present over the collected haemolymph was done with a microscope and a haemocyte counting Neubauer chamber.

After the end of 60 days, ten shrimps from each treatment were used for the *Vibrio parahaemolyticus* disease study. The bacterial strain *V. parahaemolyticus* was purchased from the College of Fisheries Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. The *V. parahaemolyticus* was cultured on tryptic soy agar plates (TSA supplemented with 2.5% NaCl) for 24 h and the culture plates was transferred to 10ml of tryptic soy broth (TSB supplemented with 2% NaCl) and was incubated at 25ºC for 24h as a stock culture for the experiment.

V. parahaemolyticus culture was centrifuged at 3000 rpm for 10 minutes at 4ºC. A PBS - *Vibrio parahaemolyticus* suspension with OD 0.8 at 620 nm which equivalent to 6.4 × 10^8 cfu/ml. This standardized bacterial suspension was centrifuged at 3000 rpm for 10 minutes and the pellet was resuspended in 50 µl of PBS. From this, 7.81 µl was made into 50 µl using PBS to get 1× 10^8 cfu/50 µL was made into 50 µL using PBS to get 1 x 10^8cfu/50 µL. These bacterial suspensions were serially diluted to get the required concentration of 10^8 to 10^4cfu/50 µL. A 100 µl of bacterial suspension in a 26 gauge number syringe containing 10^4cfu/ ml PBS (LD_{50} dose) injected over the second and third abdominal segment. The study was conducted by stocking ten shrimp in each tank. Then, ten shrimps from each CIF treatment were injected the bacterial suspension and the control treatment shrimps injected with the same concentration of PBS in triplicate. The susceptibility was conducted for 10 days separately and fed with a controlled diet. The mortalities were recorded down and removed from the respective tanks.

The collected growth performance, immune response and diseased mortalities were analyzed statistically by using one-way analysis of variance technique (ANOVA) in all the treatments. For comparison of different treatments, Duncans's multiple range tests were used. All the data were expressed as mean±standard error with a statistical significant value of $p<0.05$.

Results and Discussion

Throughout 60 days, the experimental days the water parameters were monitored onweekly basis. The optimum basic parameters for the growth and survival activity were as of Temperature (29.88±0.21°C), pH (7.35±0.21), Dissolved oxygen (5.89±0.18 mg/l) and Alkalinity (123.80±1.26 mg $CaCO_3$/ l).

Growth and survival of *Penaeus vannamei* fed *Cystoseira indica* fucoidan extract supplemented diets

Weight Gain (WG), Feed Conversion Ratio (FCR), Specific Growth Rate (SGR) and Protein Efficiency Ratio (PER) were significant difference ($p<0.05$) between the treatment groups in Table 2. There was no significant ($p>0.05$) survival rate among the treatment group in Table 2. A higher weight gain (202.8% for 500mgkg^{-1} and 215.1% for 1000 mgkg^{-1}); higher SGR (8 - 7.9% day^{-1}) was observed in the optimum range of 500-2000 mgkg^{-1} and higher survival rate was observed against *Vibrio harveyii* on the effectiveness of *Undaria pinnatifida* fucoidan extracted diet supplementation (Trifalgar *et al.,* 2009, 2010, 2012) [33, 31, 32]. The efficient protein utilization was observed in the polysaccharides *Macrocystis pyrifera* diet supplemented in *P. vannamei* juveniles (Cruz-Suárez *et al.,* 2000; Immanuel *et al.,* 2010) [7, 15].

Table 2: Growth performance and survival of *P. vannamei* fed with *Cystoseira indica* extracted fucoidan supplemented diets during the experiment period (Mean ± SE)

	Fucoidan supplementation levels (mg/kg)			
	0	**500**	**1000**	**2000**
Initial body weight (g)	0.012 ±0.00	0.012±0.00	0.012 ±0.00	0.012±0.00
Final body weight (g)	1.707 ±0.00^a	2.088±0.05^b	2.752 ±0.01^c	3.849±0.01^d
WG (g)	1.695 ±0.00^a	2.076±0.05^b	2.740 ±0.01^c	3.837±0.01^d
FCR	1.710 ±0.01^d	1.620±0.03^c	1.474 ±0.01^b	1.299±0.01^a
SGR (%)	2.826 ±0.01^a	3.460±0.09^b	4.566 ±0.01^c	6.395±0.01^d
PER (%)	1.671 ±0.01^a	1.765±0.03^b	1.938 ±0.01^c	2.199±0.02^d
Survival (%)	91.67 ±1.67^a	93.33±1.67^a	93.33 ±3.33^a	98.33±1.67^a

Mean values with different superscripts are significantly different ($p<0.05$)

Immune parameters of *Penaeus vannamei* fed *Cystoseira indica* fucoidan extract supplemented diets

Experimental shrimps fed with 2% *C. indica* fucoidan extract were shown significantly ($p<0.05$) higher phenoloxidase activity (0.192±0.00^d), higher respiratory burst activity (0.250±0.00^d) and higher haemocyte count (8.930±0.02^d) as compared to all the other treatment groups. The immune response results were listed in Table 3. A significant ($p<0.05$ higher prophenoloxidase activity, respiratory burst and total haemocyte count in experimental vannamei shrimps as compared to control shrimps when challenged with WSSV test (Immanuel *et al.,* 2012) [20].

According to Takahashi *et al*. (2000) [30], incorporating lipopolysaccharides derived from *Pantoea agglomerans* into the diet of *Marsupenaeus japonicus* significantly stimulated phenoloxidase activity and phagocytosis, thereby enhancing resistance to penaeid acute viremia virus. An elevated respiratory burst activity in groupers treated with sodium alginate (20 mg kg^{-1}) or κ-carrageenan (30 mg kg^{-1}) (Cheng *et al.,* 2008) [6].

Disease challenged against *Vibrio parahaemolyticus* infection of *Penaeus vannamei* fed *Cystoseira indica* fucoidan extract supplemented diets

The mortality of *P. vannamei* was observed after 10 days of *Vibrio parahaemolyticus* challenge test and the results were listed in Table 4. The highest mortality was observed in the control T_0 group, while the mortality was lowest in T_3 treatment shrimp fed with 2% *C. indica* fucoidan extract. Statistical analysis of data revealed that there was no significant difference between T_2 and T_3 treatments. Treatment T_0 showed significantly higher ($p<0.05$) mortality as compared to the other treatments.

The effectiveness of *Undaria pinnatifida* fucoidan supplementation in improving the survival of *Penaeus japonicus* larvae challenged with *Vibrio harveyii*. It revealed that post-challenge survival rates increased with higher dietary fucoidan supplementation. The significant ($p<0.05$) higher survival was recorded in the group receiving 1000 mg kg^{-1} fucoidan, followed by the 500 mg kg^{-1} supplementation group (Traifalgar *et al.,* 2012) [32].

A similar investigation on the effect of brown fucoidan seaweed in various shrimps which stimulated a better growth and innate immune response against *Vibrio* species and WSSV (Huang *et al.,* 2006; Traifalgar *et al.,* 2010; Kitikiew *et al.,* 2013; Sivagnanavelmurugan *et al.,* 2014; Arizo *et al.,* 2015 and Sinurat *et al.,* 2016) [13, 31, 16, 26, 3, 25].

Table 3: Immune response of *P. vannamei* fed with *Cystoseira indica* extracted fucoidan incorporated diets at the end of experiment period (Mean ± SE)

	Fucoidan supplementation levels (mg/kg)			
	0	500	1000	2000
PO activity	0.153 ± 0.00^a	0.163 ± 0.00^b	0.173 ± 0.00^c	0.192 ± 0.00^d
Respiratory Burst (NBT) activity	0.199 ± 0.00^a	0.228 ± 0.00^b	0.235 ± 0.00^c	0.250 ± 0.00^d
THC ($x10^6$ ml^{-1})	6.350 ± 0.04^a	7.507 ± 0.01^b	7.973 ± 0.03^c	8.930 ± 0.02^d

Mean values with different superscripts are significantly different ($p<0.05$)

Table 4: Mortality rate (%) of *P. vannamei* fed with *C. indica* extracted fucoidan incorporated diets at the end of the *V. parahaemolyticus* challenge experiment period (Mean ± SE)

	Fucoidan supplementation levels (mg/kg)			
	0	500	1000	2000
Mortality rate (%)	73.33 ± 3.33^d	46.67 ± 3.33^c	23.33 ± 3.33^b	13.33 ± 3.33^a

Mean values with different superscripts are significantly different ($p<0.05$).

Conclusion

The dietary inclusion study of 2% fucoidan extract derived from *Cystoseira indica* significantly enhances the growth performance of *Penaeus vannamei*, as evidenced by improved weight gain, specific growth rate (SGR) and protein efficiency ratio (PER). It also strengthens immune parameters, including increased phenoloxidase activity, respiratory burst activity, and total haemocyte count. Moreover, the treatment improves feed utilization by reducing the feed conversion ratio (FCR) and lowers mortality during a *Vibrio parahaemolyticus* challenge. Nonetheless, survival rates across treatments showed no significant differences ($p>0.05$) after 60 days of the feeding trial.

Conflict of interest

Authors have declared that there was no competing interests exist.

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